The Glucagon-sensitive Adenyl Cyclase System in Plasma Membranes of Rat Liver

III. BINDING OF GLUCAGON: METHOD OF ASSAY AND SPECIFICITY

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SUMMARY

The initial reaction between glucagon and the glucagon-sensitive adenyl cyclase system in rat liver plasma membranes is probed with the use of ^{125}I-glucagon. An assay system is described for the binding of labeled glucagon to membranes.

Iodination of glucagon with I_2 yields mono-iodo glucagon having the same biological activity as native glucagon. ^{125}I-Glucagon of high specific activity used for studying binding cochromatographs with native glucagon and behaves as native glucagon according to the following observations. (a) Biological activity and binding of labeled glucagon are reduced in parallel and to the same extent by a glucagon inactivating process in the liver membrane preparation; at high membrane concentrations, all of the medium glucagon is inactivated rapidly; (b) binding of labeled glucagon is reduced specifically by native glucagon in proportion to the relative concentration of labeled and unlabeled glucagon added; and (c) binding of glucagon is not altered in the presence of biologically inactive peptide fragments of glucagon (Fragments 1–21 and 22–29), secretin, adrenocorticotropin, and insulin. Products of glucagon degradation formed during iodination are also biologically inactive and do not bind to liver membranes.

Liver plasma membranes bind 20 times more labeled glucagon than do fat cell ghosts which contain a glucagon-sensitive adenyl cyclase system that has apparent affinity for glucagon 25-fold less than the liver membrane system (4 × 10^{-9} M glucagon). The apparent affinity of the liver membrane binding sites for glucagon is approximately the same as that of the adenyl cyclase system for glucagon. The binding sites for glucagon are finite in number (estimated 2.6 pmoles per mg of membrane protein) and are saturated with hormone in the range of 4 to 8 × 10^{-8} M glucagon, the same range found for maximal activation of adenyl cyclase by the hormone. Treatment of liver membranes with phospholipase A, digitonin, and urea cause parallel losses in binding of glucagon and activation of adenyl cyclase by the hormone. Combined with the similar range of concentrations over which glucagon binds and activates adenyl cyclase, the correlations between loss of binding and activation of adenyl cyclase by the hormone suggest that the binding sites for glucagon are related to the adenyl cyclase system.

Constant levels of bound labeled glucagon are attained within 10 min at 30°; the amount bound is markedly reduced at 0°; 2 M urea causes complete release, with full retention of biological activity, of bound labeled glucagon. Addition of unlabeled glucagon (5 μM) results in release of less than 2% of bound labeled hormone in 15 min; EDTA (1 mM), in the presence of unlabeled hormone, stimulates release of 35% of bound labeled hormone during this time.

These studies provide evidence that glucagon binds to specific sites, finite in number, that are related to the adenyl cyclase system in rat liver plasma membranes. The sites have characteristics of a lipoprotein and form a noncovalent associative-type bond with glucagon. Temperature dependence of binding and enhancement of release of bound hormone by EDTA suggest that binding may be influenced by factors in addition to the intrinsic binding forces between hormone and binding site.

The preceding studies (1, 2) described a number of characteristics of a glucagon-sensitive adenyl cyclase system in plasma membranes of rat liver. Based on the selective inhibitory effects of such agents as phospholipase A, detergents, ions, and urea on the response of the enzyme system to glucagon, it was suggested that glucagon activates the enzyme through its reaction with a specific recognition component, termed "discriminator," that may be molecularly distinct from adenyl cyclase. The same process of hormonal activation of the enzyme has been suggested for the fat cell adenyl cyclase system which, in the rat, is activated by six hormones (glucagon, secretin, adrenocorticotropin, thyrotropin, luteinizing hormone, and catecholamines), each of which activates a common enzyme system through specific recognition sites or discriminators (3, 4).

The discriminator has two functions. One is to recognize and combine with its specific hormone, the other is translation of this reaction into activation of adenyl cyclase. The properties of

both functions must be known in order to understand the mode of action of hormones on the adenyl cyclase system. Insight into the possible nature of the initial reaction between hormone and discriminator stems from the recent report (5) that ACTH[1] binds specifically and reversibly with membranes from adrenal cortical cells containing an adenyl cyclase system specific for ACTH. It has also been reported (6) that glucagon binds to plasma membranes of rat liver, although no evidence of specificity, reversibility of binding, or relation of binding to adenyl cyclase activation by the hormone was presented.

In the present study, an approach similar to that described for the binding of ACTH has been taken for studying the reaction between glucagon and liver plasma membranes, with the following objectives: to establish that labeled glucagon behaves as native glucagon with respect to the binding reaction; to determine both the specificity of binding and the possible relationship of the binding sites to the adenyl cyclase system; and to characterize some of the properties of the binding process. Subsequent studies (7, 8) will report our attempts to relate the binding reaction to the process of activation of adenyl cyclase. Some of the studies have been reported elsewhere in preliminary form (9).

EXPERIMENTAL PROCEDURES

Materials

Carrier-free $Na^{125}I$ (in 0.1 N NaOH) was obtained from Union Carbide Company. $ATP\text{-}\alpha\text{-}^{32}P$ was supplied by the International Chemical and Nuclear Corporation and was purified further as described elsewhere (1). Purified glucagon (mixture of porcine and bovine), bovine insulin, and a fragment of glucagon comprising the NH_2-terminal 1–21 portion were generously supplied by Dr. Otto Behrens of Eli Lilly and Company. A carboxy-terminal fragment of glucagon (Fragment 22–29) was kindly supplied in purified form by Dr. Victor Hruby of the University of Arizona, Tucson. Highly purified porcine secretin was a gift of Dr. Victor Mutt of the Karolinska Institute, Stockholm. Highly purified phospholipase A was described in the previous report (2). Bovine serum albumin (Fraction V, lot E-29907) was purchased from Armour and Company. All other reagents and hormones were of the highest grade of purity obtainable. Plastic micro test tubes (0.4-ml capacity) were purchased from Beckman (catalogue no. 314321).

Methods

Preparation of Hepatic Plasma Membranes—Plasma membranes were isolated from livers of female rats (Charles River, 120 to 140 g) by the procedure of Neville (10) with the exception that the last stage of purification (rate zonal centrifugation) was omitted (partially purified membranes (1)). No detectable change in either adenyl cyclase activity and its response to glucagon or of the characteristics of binding of glucagon to the membranes was observed after at least 3 months of storage in liquid nitrogen.

Preparation of Fat Cell Ghosts—Ghosts of isolated fat cells, prepared from adipose tissue of the same rats used for isolating liver plasma membranes, were isolated as described previously (11). At the final stage of preparation (washed with 1 mM $KHCO_3$ and centrifuged), pellets of ghosts were suspended in

[1] The abbreviation used is: ACTH, adrenocorticotropin.

20 mM Tris-HCl, pH 7.5, containing 1 mM EDTA and were immediately frozen and stored under liquid nitrogen. Adenyl cyclase activity and sensitivity of the enzyme system to glucagon was retained in preparations of ghosts stored in this manner in contrast to the marked decrease in activity of the enzyme previously reported for ghosts kept at 0° for only a few hours (12).

Preparation of Iodinated Glucagon (I_2 *Method*)—Glucagon (3.5 mg, 1 μmole), dissolved in 0.1 ml of dimethylsulfoxide, was mixed with 0.2 ml of 0.2 N sodium acetate, pH 5.5, containing approximately 300 μCi of $Na^{125}I$ (carrier-free). To this solution were added, with rapid mixing, 15 μl of CCl_4 containing 1.0 μmole of I_2. The mixture was shaken mechanically for 3 min at room temperature, after which 0.2 ml of dimethylsulfoxide was added to increase the density of the solution. The mixture was layered, with the aid of a Pasteur pipette, directly above a preparative column of 4% acrylamide gel, described below, that was used for separating iodinated from noniodinated glucagon.

Gel electrophoresis of iodinated glucagon was performed in a Fractophorator apparatus obtained from Buchler Instruments, Fort Lee, New Jersey. With the exception that urea was omitted from all solutions, lower and upper gels were prepared using the gel solutions described by Reisfeld and Small (13). The length of the lower gel column was 10 cm; that of the upper gel, 1.5 cm. The buffer (pH 8.9) in the upper chamber consisted of 43 mM Tris and 46 mM glycine. The buffer (pH 8.1) in the lower chamber consisted of 120 mM Tris and 60 mM HCl.

After layering the sample above the column, electrophoresis was started using a constant current of 25 ma in a 5° refrigerated room. Elution was achieved using the same buffer (pH 8.1) described for the lower chamber. Fractions were collected with an automatic fraction collector set to collect 2.2 ml every 10 min. The eluted fractions were analyzed spectrophotometrically for protein by measuring the absorbance at 280 mμ (silica cuvette, 1-cm light path).

Preparation of High Specific Activity ^{125}I-*Glucagon* (*Chloramine-T Method*)—The procedure used was a slight modification of the method originally described by Hunter and Greenwood (14) for iodinating peptide hormones. All reagents were dissolved in 0.6 M sodium phosphate buffer, pH 7.4. Stock solutions of glucagon (3 to 3.5 mg per ml) were prepared by dissolving the hormone, with heating at 60°, in 0.5 M Tris-HCl, pH 8.5. Concentrations of glucagon were determined on the basis of a molar extinction coefficient of 8050 M^{-1} cm^{-1} at 280 mμ (15). Just prior to iodination the stock solution of glucagon was diluted in the phosphate buffer to give a concentration of 350 μg per ml. Then 10 μl of this solution (1.0 nmole) were placed at the bottom of a plastic micro test tube (0.4-ml capacity). On the sides of the tube were placed 5 μl of a solution of $Na^{125}I$ (1.0 to 2.0 mCi, about 0.5 to 1.0 natom) and 10 μl of a freshly prepared solution of chloramine-T (3.5 mg per ml). The iodination reaction was initiated by mixing the reagents rapidly with the aid of a Vortex mixer. After 15 sec, the reaction was stopped by the addition, again with rapid mixing, of 50 μl of a solution of sodium metabisulfite (2.4 mg per ml). Exposure times longer than 15 sec resulted in losses of 80 to 90% of active glucagon as assayed by activation of adenyl cyclase.

^{125}I-Glucagon was purified on a column of cellulose powder (for thin layer chromatography, Arthur H. Thomas Company) by a slight modification of the method described by Yalow and Berson (16) for purifying iodinated insulin. The column (0.4 ml) was

prepared in a Pasteur pipette (0.6 cm inner diameter) and washed repeatedly with a solution of 1% albumin in 10 mM sodium phosphate buffer, pH 7.4. After application of the reaction mixture described above, the column was eluted with the same solution of albumin-phosphate buffer until the eluates showed little radioactivity (usually after the passage of 3.0 ml of eluting solution). At this point, the column was eluted with 0.5 ml of a solution of 1% albumin adjusted to pH 10 with concentrated ammonium hydroxide. This fraction contained 30 to 40% of the radioactivity and of the biologically active glucagon applied to the column. The concentration of glucagon eluted in this fraction was determined by assaying serially diluted solutions for stimulation of adenyl cyclase activity (in liver membranes) and comparing the activities with standard solutions of native glucagon.

Chromatography of Labeled Glucagon—Portions of ^{125}I-glucagon preparations (chloramine-T method) were analyzed for radiopurity by thin layer and ion exchange chromatography. For thin layer chromatography, 10 μl of labeled material was co-chromatographed with 10 μg of native glucagon on precoated glass plates (20 × 20) of Avicel microcrystalline cellulose powder obtained from Brinkmann. The plates were developed by ascending flow using the butanol-pyridine-water-glacial acetic acid (30:20:24:6) solvent system described by Brewer *et al.* (17). The plates were stained with ninhydrin; radioactive material was detected by radioautography on Kodak no-screen x-ray plates.

Ion exchange chromatography of ^{125}I-glucagon was carried out on columns (0.8 × 4.5 cm) of DEAE-cellulose (Whatman DE52) equilibrated with 0.01 M Tris-HCl, pH 7.6. Samples of labeled or unlabeled glucagon (or both) were applied in 0.1 ml of starting buffer and were eluted with 30 ml of a linear gradient formed from equal volumes of 0.01 and 0.5 M Tris-HCl, pH 7.6. Chromatography was carried out at room temperature and at a flow rate of 0.3 ml per min. Biologically active glucagon in the fractions obtained was assayed by the adenyl cyclase procedure described below.

Measurements of Adenyl Cyclase Activity—Adenyl cyclase activity was measured by the conversion of ATP-α-^{32}P to cyclic 3',5'-AMP, using the same incubation medium described previously (1). Adenyl cyclase activity is expressed as nanomoles of cyclic 3',5'-AMP formed in 10 min per mg of protein.

Measurement of Binding of ^{125}I-Glucagon to Plasma Membranes—Incubations were carried out at 30° in glass test tubes (10 × 75 mm). The incubation medium contained 2.5% albumin, 1 mM EDTA, 20 mM Tris-HCl, pH 7.6, and ^{125}I-glucagon at the concentrations stated in legends to figures and tables. Liver membranes (20 to 50 μg of protein), suspended in a solution containing 2.5% albumin and 20 mM Tris-HCl, pH 7.6, were added to complete a reaction volume of 125 μl. After 15 min of incubation, two methods were used to separate membrane-bound from free ^{125}I-glucagon. Method A was as follows. Duplicate aliquots (25 μl or 50 μl) of the incubation mixture were layered over 0.3 ml of a solution of 2.5% albumin in 20 mM Tris-HCl, pH 7.6, contained in plastic micro test tubes. The tubes were centrifuged immediately in a Beckman microcentrifuge (catalogue no. 314300) for 5 min in a 5° refrigerated room. The supernatant fluid was aspirated with the aid of a 22-gauge needle attached to a vacuum line; the needle was cut so that its blunted end projected approximately 1 mm above the pellet in the tip of the centrifuge tube. The pellet was

washed by adding 0.3 ml of a 10% solution of sucrose (without disturbing the pellet), and then aspirating the fluid as described above. The tip of the centrifuge tube was cut off with a razor blade at a point just above the pellet and was transferred to a plastic tube for the purpose of counting. Visualization of the small pellet of membranes was aided by shining a high intensity light on the centrifuge tube lying on a piece of blackened cardboard.

The adequacy of the centrifugation was checked by centrifuging identical tubes in the microfuge and at 200,000 × g_{av} in a preparative ultracentrifuge. The high force centrifugation was achieved by inserting microfuge tubes in adapters (Beckman catalogue no. 305527), filling all unoccupied space in the adapter with water to avoid deformation of the tube, and centrifuging in a SW 50.1 rotor for 30 min at 50,000 rpm. Under standard assay conditions, the radioactivity sedimented at the two forces was identical.

As a means of correcting for the quantity of unbound ^{125}I-glucagon in the pellet, an equivalent amount of incubation medium containing the same concentration of labeled glucagon but without liver membranes was treated as described above for the test samples. The quantity of radioactivity in the tips of the centrifuge tubes was subtracted from that found in tubes containing membranes; this difference is referred to as bound glucagon.

Method B was used primarily when high concentrations (>2 × 10^{-8} M) of labeled glucagon were added to the incubation medium and was prompted by the finding that, using Method A, the difference in radioactivity between the control (no membranes) and the membrane pellets was too small for accurate assessment of the net amount of bound labeled hormone. This method involved layering the incubated samples (0.2 ml) over 5 ml of 20% sucrose in cellulose nitrate tubes and centrifuging in the SW 50.1 rotor for 15 min at 50,000 rpm. Using this procedure, in which only six samples could be centrifuged at one time, labeled glucagon sedimented only in tubes containing membranes.

Determinations of ^{125}I were performed in a well type scintillation γ counter.[a] In most experiments, the quantity of bound glucagon is expressed either as the percentage of radioactivity added to the incubation medium or, calculated from the specific activity of ^{125}I-glucagon, as picomoles of glucagon bound per mg of membrane protein added to the incubation medium.

Membrane protein was determined by the procedure of Lowry *et al.* (18) after dissolving the membranes, with heating in a boiling water bath, in 0.1 ml of 1 N NaOH. Crystalline bovine serum albumin was used as standard.

Replicate determinations were made in all binding experiments; each experiment was carried out at least twice, in some cases with different batches of membranes and labeled glucagon as specified in legends to figures and tables.

RESULTS

Preparation and Biological Activity of ^{125}I-Glucagon

I₂ Method—Glucagon labeled with radioactive iodine has been employed successfully for radioimmunoassay of the hormone in blood (19), indicating that iodinated glucagon binds to glucagon antibodies in a manner similar to that of native glucagon. For this reason and because of the relative ease of preparing iodinated glucagon, ^{125}I-glucagon (referred to henceforth as
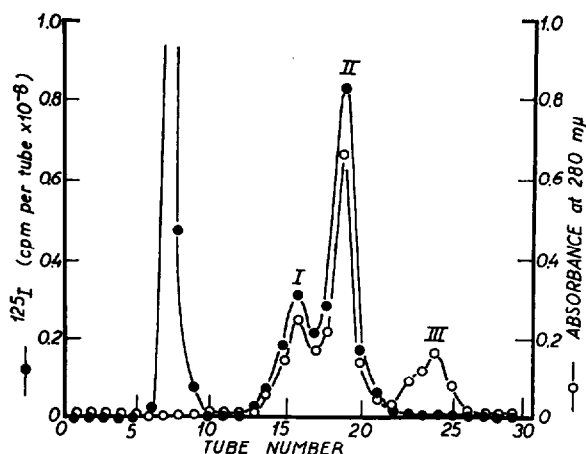
FIG. 1. Separation of iodinated from noniodinated glucagon by preparative gel electrophoresis. Procedures for iodination of glucagon (I₂ method) and for electrophoresis on 4% acrylamide gel columns are described under "Methods."
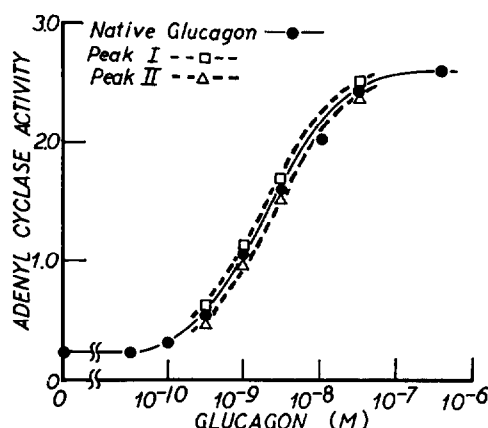

FIG. 2. Dose response curves given by iodinated and native glucagon on adenyl cyclase activity in hepatic plasma membranes. Iodinated glucagon was prepared and separated from uniodinated glucagon as described under "Methods" (I₂ method). The peak tubes collected in Fractions I and II (see Fig. 1) were assayed for glucagon content by measuring their effects on adenyl cyclase activity compared to native glucagon at the indicated concentrations. Concentrations of protein in Fractions I and II were estimated from their absorbance at 280 mμ using an extinction coefficient for glucagon of 8050 M^{-1}. Adenyl cyclase activity was measured under conditions described under "Methods" and by an assay procedure described elsewhere (12) in detail.

labeled glucagon) seemed to be appropriate material for studying the reaction between the hormone and hepatic plasma membranes.

The first point to be established was whether incorporation of iodine into the tyrosines of glucagon would alter the biological activity of the hormone. For this purpose, glucagon was iodinated with I₂ since it has been reported (20) that this method, particularly at a pH less than neutrality, leads to less cleavage and oxidation of peptides relative to methods using stronger oxidants. A relatively large amount (3.5 mg) of glucagon was iodinated with I₂ containing ^{125}I for purpose of quantitation and identification of the labeled reaction products. A maximum of 50% of the iodine (or ^{125}I) can be incorporated into glucagon under these conditions. The products were subjected to electrophoresis on an acrylamide gel column. As shown in Fig. 1,

three labeled peaks were obtained. The first peak contained free iodine and accounted for 57% of the radioactivity added during the iodination reaction. The remaining 43% of the radioactive iodine was recovered in the iodinated protein fractions, designated as Fractions I and II, which comprised approximately 80% of the total protein recovered and 86% of the theoretical yield of iodinated material. These two fractions represent, therefore, mostly glucagon having an average of 1 iodine atom per molecule of glucagon. It is likely that the smaller of the two iodinated fractions represents desamido-glucagon which is present in preparations of glucagon (21) and which migrates faster toward the anode because of its higher negative charge (22). The fact that iodinated glucagon migrates faster than native glucagon (Peak III, noniodinated) is attributed to increased ionization of the tyrosylhydroxy group upon iodination of the tyrosine ring.

Based on the amount of protein in the peak tubes of the iodinated material, these fractions were assayed for biologically active glucagon using the adenyl cyclase system in hepatic membranes. It can be seen in Fig. 2 that Fractions I and II yielded dose response curves that were indistinguishable from that given by native glucagon, indicating that incorporation *per se* of iodine into the tyrosines of glucagon does not alter the biological activity of the hormone.

Chloramine-T Method—The I₂ method of iodination, when applied on a nanogram scale, did not yield high specific activity ^{125}I-glucagon. ^{125}I-Glucagon used for the binding studies was prepared by the chloramine-T method of Hunter and Greenwood (14). A large fraction (60 to 70%) of the added glucagon was inactivated even during brief exposure of the hormone to chloramine-T.

Purification of labeled glucagon was achieved by taking advantage of the fact, previously noted by Yalow and Berson (16), that glucagon adsorbs strongly to cellulose. Biologically active hormone was eluted from cellulose columns only after application of 1% albumin, pH 10, as described under "Methods." More than 95% of the eluted radioactive material co-chromatographed with glucagon on thin layer cellulose chromatography (17) or eluted as a single peak with native hormone during chromatography on DEAE-cellulose. During development of the purification procedure, it was observed that failure to equilibrate the cellulose column with 1% albumin (pH 7.5) prior to application of iodinated reaction products, resulted in adsorption of labeled material which, after elution at pH 10 with active hormone, appeared as a separate peak from iodinated glucagon during DEAE-cellulose chromatography. This material, which comprised about 40% of the total ^{125}I incorporated into reaction products, did not bind to liver membranes and was not biologically active.

Yields of biologically active material ranged from 30 to 40% of that added during iodination, with specific activities ranging from 0.5 to 1.0 × 10⁶ cpm per pmole of glucagon assayed by adenyl cyclase activity. Based on the efficiency of the γ counter (35%), the highest specific activity obtained was about 50% of the theoretical maximum of 2.4 μCi per pmole of glucagon if 1 atom of iodine is incorporated per mole of hormone.

Attempts to separate labeled from unlabeled glucagon by the gel electrophoresis procedure described above proved unsuccessful, primarily because it was found that glucagon treated with chloramine-T migrates as rapidly as iodinated glucagon, possibly because of deamidation of glucagon.

Binding of ¹²⁵I-glucagon to hepatic plasma membranes

Reaction mixtures contained 2.5% albumin, 1 mM EDTA, 20 mM Tris-HCl, pH 7.6, 4 × 10⁻⁹ M ¹²⁵I-glucagon (10⁶ cpm per pmole), and 0.4 mg per ml of plasma membrane protein. Final volume was 0.125 ml, temperature 30°. After incubation for 15 min, 25-μl samples were layered over 0.3 ml of 2.5% albumin in 20 mM Tris-HCl, pH 7.6, contained in plastic centrifuge tubes. The tubes were centrifuged for 5 min at 10,000 × g, the supernatant fluid aspirated, and the membrane pellets washed with 0.3 ml of 10% sucrose. The tips of the tubes were cut off and counted as described under "Methods." Controls (no membranes, same medium) were treated as above. The difference between test samples (membranes added) and control is bound glucagon. The percentage of glucagon bound was calculated as follows:

$$\% \text{ bound} = \frac{\text{bound glucagon (cpm)} \times 100}{\text{total glucagon (cpm)}}$$

The results were obtained with two preparations of plasma membranes and are the mean ± standard error of the quantity of radioactivity found in the tips of the centrifuge tube in four determinations.

Membrane preparation	¹²⁵I-Glucagon in tips of centrifuge tubes		Bound glucagon
	−Membranes	+Membranes	
	cpm		%
I	578 ± 28	9,858 ± 650	9.4
II	635 ± 27	10,340 ± 589	9.7

Reaction of Labeled Glucagon with Hepatic Membranes

Binding Assay—Binding of labeled glucagon to plasma membranes was assayed by first incubating membranes with labeled hormone in medium containing albumin, EDTA, and Tris buffer, pH 7.6. Albumin was required in the assay medium to minimize nonspecific adsorption of the hormone to glass and plastic. EDTA (1 mM) was included in the incubation medium because of our finding (1) that it enhances the effect of glucagon on adenyl cyclase activity in liver membranes. As described under "Methods," membrane-bound hormone was separated from free labeled hormone by centrifuging the suspension of membranes at approximately 10,000 × g for 5 min, washing the pellet with a solution of 10% sucrose, and then cutting off the tip of the centrifuge tube just above the pellet of membranes. This procedure removed at least 99% of the free labeled glucagon as shown by the experiments reported in Table I in which only 0.5% of the added labeled glucagon was found in tips of control tubes (no membranes added); approximately 10% of the labeled hormone, at 4 × 10⁻⁹ M, was taken up by the membranes in these experiments, with variations of less than 1% with two different preparations of membranes.

Comparison of Binding of Labeled Glucagon to Fat Cell Ghosts and Liver Membranes—Ghosts of fat cells also contain an adenyl cyclase system that is activated by glucagon (3, 4). The concentration of glucagon giving half-maximal activation of ghost adenyl cyclase is approximately 10⁻⁷ M (3), or about 25-fold higher than that observed with liver membrane adenyl cyclase (see Fig. 8). Binding of labeled glucagon, under identical assay conditions, to the two membrane preparations is illustrated in Fig. 3. In the presence of 4 × 10⁻⁹ M labeled glucagon, less than 2% of the hormone was taken up by ghosts at a concentration
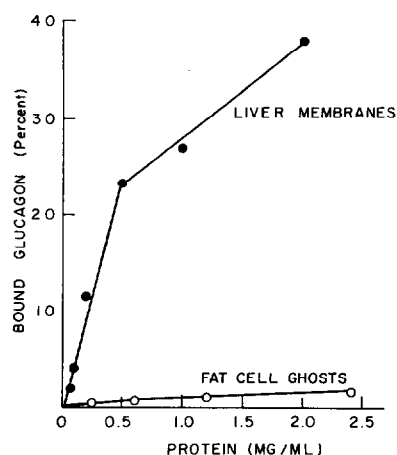

FIG. 3. Binding of labeled glucagon to fat cell ghosts and liver plasma membranes. Liver plasma membranes and fat cell ghosts, prepared as described under "Methods," at indicated concentrations of protein, were incubated at 30° in a final volume of 0.125 ml of medium containing 2.5% albumin, 1 mM EDTA, 20 mM Tris-HCl, pH 7.6, and 4.5 × 10⁻⁹ M ¹²⁵I-glucagon. Separation of membrane-bound from free labeled glucagon was carried out by Method A under "Methods."

of 2.4 mg of protein per ml. In contrast, liver membranes bound 2% of the labeled hormone at 0.1 mg of membrane protein per ml and 38% at 2.0 mg of protein per ml. The marked difference in binding of the hormone by liver and fat cell membranes is consistent with the differences in sensitivity of their adenyl cyclase systems to glucagon. Detailed studies of the binding of glucagon to fat cell ghosts are in progress. The results of these comparative studies with different membrane preparations indicate that the method for assaying binding of glucagon is a measure of binding that is a function both of the concentrations and properties of the membranes.

Inactivation of Glucagon by Liver Membranes—It will be noted in Fig. 3 that binding of glucagon to liver membranes was proportional to membrane protein concentration up to 0.5 mg per ml. A maximum of 38% of the labeled hormone was bound at the highest concentration of liver membrane protein tested (2 mg per ml). It has also been found that activation of adenyl cyclase by glucagon is only proportional to membrane concentration within the range of 0.1 to 1.0 mg of protein per ml (1). The possibility that decreased binding and activation of adenyl cyclase at higher concentrations of membranes reflected inactivation or destruction of the hormone was examined either by following the disappearance of bindable labeled glucagon or biologically active hormone. Both methods involved incubating the liver membranes with labeled hormone under standard conditions, removing the membranes by centrifugation, and then assaying the supernatant fluid for either binding of labeled material or activation of adenyl cyclase in fresh liver membranes. Bindable or biologically active glucagon remaining after the first incubation was determined from standard curves for binding and activation of adenyl cyclase given by various concentrations of the labeled hormone. As shown in Fig. 4, glucagon was inactivated rapidly, nearly 50% of the hormone being destroyed with respect to binding or activation of adenyl cyclase within the first 2 min of incubation with 0.8 mg of membrane protein per ml. The finding that both methods of assay gave identical curves of inactivation is evidence that the labeled hormone is an
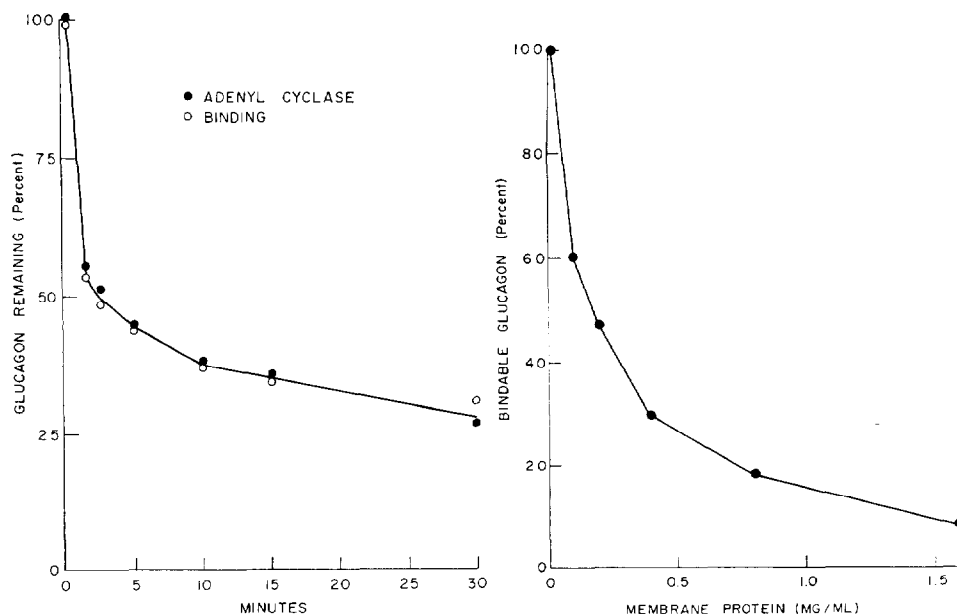
Fig. 4 (*left*). Time course of inactivation of ^{125}I-glucagon with respect to binding and biological activity (adenyl cyclase assay). Liver membranes, 0.25 mg per ml, were incubated in 0.3 ml of medium containing 2.5% albumin, 20 mM Tris-HCl, pH 7.6, 1 mM EDTA, and 5 × 10⁻⁹ M labeled glucagon (10⁶ cpm per pmole). Incubations were at 30° for the indicated times. The incubation mixture was transferred to microfuge tubes and centrifuged for 5 min at 10,000 × *g*. An aliquot (0.1 ml) of the supernatant was added to 0.025 ml of incubation medium (as above) and fresh liver membrane to give a final concentration of 0.25 mg of membrane protein per ml. After incubation for 15 min, bound glucagon was assayed by Method A (see "Methods"). Duplicate 0.025-ml aliquots of the remaining supernatant were added to tubes containing adenyl cyclase assay reagents and assay for ability to stimulate adenyl cyclase activity under previously described conditions (1). Using a standard curve of either binding or adenyl cyclase activa-

tion *versus* glucagon concentration, the specific activity (counts per min per pmole) of glucagon in each of the supernatants was determined. The increase in the apparent specific activity due to incubation with membranes was attributed to inactivation of labeled glucagon. The results were calculated according to the following equation:

$$\text{Glucagon remaining} = \frac{\text{specific activity of nonincubated glucagon} \times 100\%}{\text{specific activity of incubated glucagon}}$$

Fig. 5 (*right*). Effect of liver membrane concentration on inactivation of ^{125}I-glucagon. Varying amounts of liver membranes were incubated for 10 min under the conditions and by the binding assay described in the legend to Fig. 4.

adequate marker for studies of the reaction of glucagon with the liver membrane system with respect to its biological activity, binding to the membranes, and inactivation.

In Fig. 5, it is seen that increasing the concentration of liver membranes to 2 mg per ml resulted in essentially complete inactivation of glucagon in the incubation medium. Studies on the specificity and other characteristics of the inactivation process are in progress.

Specificity of Binding—If the sites that bind labeled glucagon are specific for glucagon and have an equivalent affinity for both native and iodinated glucagon, only native glucagon should prevent, by dilution, the binding of the labeled glucagon in proportion to their relative concentrations in the incubation medium. As shown in Fig. 6, dilution of the labeled hormone (4 × 10⁻⁹ M) with concentrations of unlabeled hormone up to 4 × 10⁻⁹ M resulted in the expected proportional decrease in the percentage of labeled hormone bound to plasma membranes. The lack of proportional dilution at higher concentrations reflected increased uptake of glucagon as the concentrations approach saturation of the binding sites (see Fig. 8).

Specificity of the binding sites for glucagon was investigated by adding to the incubation medium purified peptide fragments of glucagon[2] (Fragments 1–21 and 22–29), and a number of

peptide hormones. As shown in Fig. 7, secretin, a peptide hormone having marked similarity in amino acid sequence to that of glucagon (23), and the two peptide fragments of glucagon did not alter the binding of labeled glucagon. Among other peptide hormones tested, ACTH and insulin also failed to alter the binding of glucagon. It will be noted that 1.0 μg per ml of (2.8 × 10⁻⁶ M) glucagon completely prevented the binding of labeled glucagon. Coupled with the dilution experiments reported above, these findings provide evidence that the sites of binding of glucagon are specific for this hormone and validate further the use of [124]I-glucagon as a label for studying the specific binding of glucagon to hepatic membranes.

Relative Dependence of Binding and Activation of Adenyl Cyclase on Glucagon Concentration—The range of concentrations over which labeled glucagon binds to liver membranes was nearly the same as that found for activation of adenyl cyclase by native glucagon (Fig. 8). The concentration of glucagon giving half-maximal binding or activation of adenyl cyclase was approximately 4 × 10⁻⁹ M. Saturation of the binding sites occurred in the range of 4.0 to 8.0 × 10⁻⁸ M, indicating that there is a finite number of sites for specific binding of glucagon to liver membranes. Based on the specific activity of the

[2] Unpublished experiments showed that the peptide fragments of glucagon (Fragments 1–21 and 22–29) did not activate adenyl

cyclase or compete with glucagon in the activation process at the concentrations indicated in these experiments.
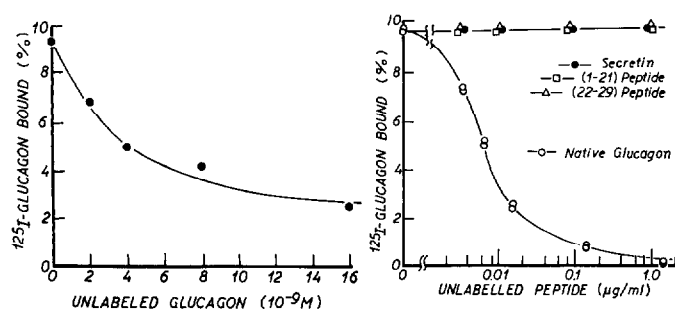
FIG. 6 (*left*). Effects of dilution of ^{125}I-glucagon with unlabeled glucagon on the binding of labeled glucagon. Plasma membranes (25 μg of protein) were incubated at 30° for 15 min in a final volume of 0.125 ml of medium containing 2.5% albumin, 1 mM EDTA, 20 mM Tris-HCl, pH 7.6, 4 × 10⁻⁹ M glucagon (10⁶ cpm per pmole), and the indicated concentrations of unlabeled glucagon. Separation of membrane-bound from free labeled glucagon was carried out as described under "Methods."

FIG. 7 (*right*). Effects of varying concentrations of glucagon, secretin, and peptide fragments (1–21 and 22–29) of glucagon on the binding of ^{125}I-glucagon to plasma membranes. Plasma membranes (60 μg of protein) were incubated at 30° for 15 min in 0.125 ml of medium containing 2.5% albumin, 1 mM EDTA, 20 mM Tris-HCl, pH 7.6, 7.0 ng of ^{125}I-glucagon (1.85 × 10⁶ cpm per pmole) per ml in the presence and absence of the indicated concentration of unlabeled glucagon, secretin, and peptide fragments of glucagon. Because of its insolubility in water, the peptide Fragment 22–29 of glucagon was dissolved in dimethylsulfoxide to give a concentration of 3.5 mg per ml and was then diluted to the indicated concentrations in the same medium used in the binding studies. It should be noted that the logarithm of the concentration of unlabeled peptides is plotted on the *abscissa*. Separation of membrane-bound from free labeled glucagon was carried out by Method A (See "Methods").

labeled glucagon, the amount of glucagon bound at saturation was calculated to be 2.6 pmoles per mg of membrane protein.

Comparative Effects of Phospholipase A on Binding of Glucagon and Activation of Adenyl Cyclase by Glucagon—In the previous report (2), it was shown that prior treatment of liver membranes with phospholipase A results in selective inhibition of the response of adenyl cyclase to glucagon, the fluoride-response being either enhanced or unaffected. As shown in Fig. 9, prior treatment with phospholipase A over the same range of concentrations, resulted also in the loss of glucagon binding. Slight enhancement of the response of adenyl cyclase to glucagon at low concentrations of phospholipase A may reflect an action on the catalytic component of the adenyl cyclase system, since the fluoride-response was also enhanced under these conditions (2). The inhibitory effects of phospholipase A were prevented completely by chelation of calcium prior to incubation of liver membranes with the calcium-dependent enzyme.

Binding of Glucagon to Digitonin-treated Membranes—In the previous study (2), it was shown that digitonin selectively inactivates the response of liver membrane adenyl cyclase to glucagon. Comparative effects of prior treatment of liver membranes with 1% digitonin on binding of glucagon and activation of adenyl cyclase by the hormone are shown in Table II. Digitonin treatment resulted in 70% loss of glucagon binding and over 90% loss of the effect of glucagon on adenyl cyclase activity.

Effects of Urea on Binding of Glucagon—It was reported previously (1) that 0.4 M urea decreased the apparent affinity of the liver adenyl cyclase system for glucagon. The effects
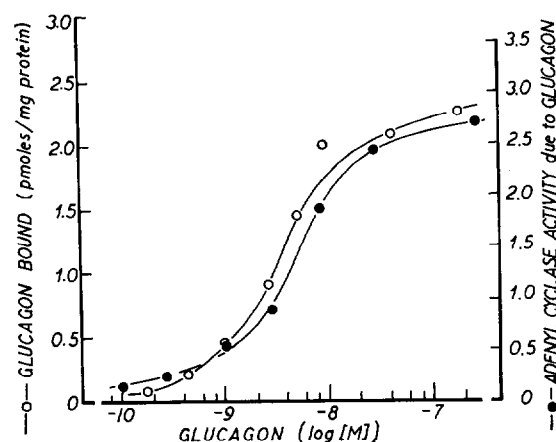

FIG. 8. Relationship between binding of labeled glucagon and response of adenyl cyclase to various concentrations of labeled and native glucagon. In the binding studies, liver plasma membranes (25 μg of protein) were incubated at 30° in 0.125 ml of medium containing 2.5% albumin, 1 mM EDTA, 20 mM Tris-HCl, pH 7.6, and the indicated concentrations of ^{125}I-glucagon (designated as glucagon for simplicity). After 15 min of incubation, bound labeled glucagon was separated from free labeled glucagon by Method A (see "Methods"). Calculations of the amount of glucagon bound were based on the specific activity of labeled glucagon (1.0 × 10⁶ cpm per pmole) and the quantity of radioactivity bound per mg of membrane protein (see "Methods"). The response of adenyl cyclase in liver membranes to glucagon was measured as follows. Liver membranes (25 μg of protein) were incubated for 10 min at 30° in 0.05 ml of medium containing 3.2 mM Tris-ATP-α-³²P, 5 mM MgCl₂, 25 mM Tris-HCl, pH 7.6. An ATP-regenerating system consisting of 20 mM phosphocreatine and creatine phosphokinase (1 mg per ml), and glucagon at the indicated concentrations. Assays of adenyl cyclase activity were carried out as described elsewhere (12) in detail. Results of the binding experiments are the average of two experiments.

of urea on the binding process were examined as another means of relating the binding sites for glucagon to the adenyl cyclase system. As shown in Fig. 10, increasing concentrations of urea caused a parallel fall in binding of labeled glucagon and activation of adenyl cyclase by native hormone.

Previous treatment of liver membranes with 2 M urea followed by dilution of the urea concentration to 0.06 M resulted in no detectable change in the binding capacity of the membranes; urea appears to cause a reversible change in the affinity of the binding sites for glucagon.

Evidence for Retention of Biological Activity by Bound Glucagon—In the course of studying the effects of urea on binding of glucagon, it was found that 2 M urea caused complete dissociation of glucagon from its binding sites. This finding afforded the means of testing whether bound glucagon retains biological activity when dissociated by the action of urea. In a typical experiment, a concentrated suspension of plasma membranes (3.2 mg of protein per ml) was incubated in the standard incubation medium for 15 min with 8.0 × 10⁻⁸ M labeled glucagon (specific activity 10⁶ cpm per pmole). The suspension (0.5 ml) was layered over 5 ml of 20% sucrose and centrifuged for 20 min at 50,000 rpm in a Beckman SW 50.1 rotor. The pellet was rinsed and suspended in fresh incubation medium; it contained 2.1 pmoles of bound glucagon per mg of protein or near-saturation of the binding sites. Urea was added to a final concentration of 2 M and the suspension (0.135 ml) was incubated for 15 min at
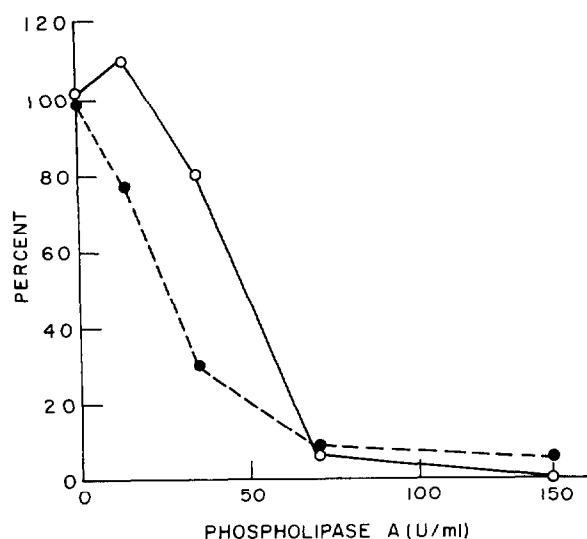
Fig. 9. Effects of phospholipase A treatment of liver plasma membranes on binding of ^{125}I-glucagon and response of adenyl cyclase to native glucagon. Plasma membranes (340 μg of protein) were incubated in 0.110 ml of 25 mM Tris-HCl, pH 7.6, containing 1 mM CaCl₂ and the indicated concentrations of phospholipase A. After 5 min at 30°, ethylene glycol bis(β-aminoethylether)-N,N'-tetraacetic acid (EGTA) was added to a final concentration of 2 mM. Aliquots of the suspension were incubated in the standard binding medium (see "Methods") containing 5 × 10⁻⁹ M ^{125}I-glucagon (10⁶ cpm per pmole) and assayed for binding using both Methods A and B (see "Methods") and in the medium described previously (1) for assaying the response of liver membrane adenyl cyclase to glucagon (10 μg per ml). Results are the average of two determinations. Addition of EGTA along with phospholipase A during first stage of incubation resulted in no loss of binding or activation of adenyl cyclase by glucagon.

TABLE II

Binding of labeled glucagon to digitonin-treated liver membranes

Liver membranes (1.8 mg of protein) were suspended in 400 μl of medium containing 25 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 1% digitonin. After 2 min at 0°, 10 ml of 25 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA were added to the suspension which was centrifuged for 30 min at 15,000 rpm in a refrigerated centrifuge. The pellet of membranes was rinsed twice with the Tris-EDTA buffer and then suspended in Tris-EDTA buffer containing 5 mM dithiothreitol. Portions of the suspension were analyzed either for glucagon-stimulated adenyl cyclase activity (final concentration of glucagon in adenyl cyclase incubation medium was 2.8 × 10⁻⁶ M) or for binding of labeled glucagon (1.7 × 10⁻⁹ M; specific activity, 10⁶ cpm per pmole) as described under "Methods." Control membranes (no digitonin treatment) were carried through the same procedures. Results are the average of two experiments.

Prior treatment	Binding of labeled glucagon	Glucagon-stimulated adenyl cyclase activity
	pmole/mg protein	*nmoles cyclic 3',5'-AMP/mg protein/10 min*
None	0.37	5.26
Digitonin	0.12	0.30

30°, which resulted in complete release of the labeled material.[3] The membranes were removed by centrifugation and the super-

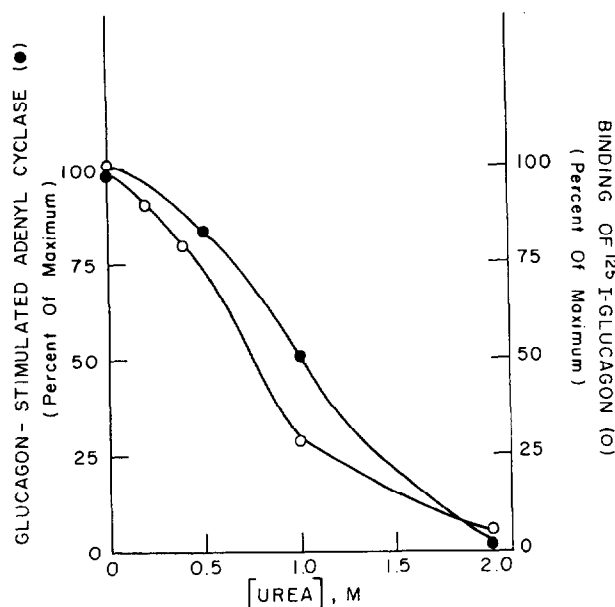[3] It was also found that 2 M urea inhibits the inactivation of glucagon by liver membranes.

Fig. 10. Effects of urea on binding ^{125}I-glucagon and activation of adenyl cyclase by native hormone. Urea was added, at the concentrations indicated, to either the standard medium for binding (see "Methods") or to the incubation described previously (1) for assaying response of adenyl cyclase to glucagon. ^{125}I-Glucagon (10⁶ cpm per pmole) in the binding assay was 4.5 × 10⁻⁹ M; in the adenyl cyclase assay medium, 10 μg per ml of native glucagon.

natant fluid was examined for glucagon activity as measured by stimulation of adenyl cyclase activity in liver membranes. The amount of radioactivity in the supernatant fluid was used as a measure of the predicted concentration of active glucagon. Adenyl cyclase activity given by the predicted concentration was compared to that given by equivalent amounts of fresh labeled glucagon incubated under identical conditions with liver membranes. In this experiment, adenyl cyclase activity given by an equivalent amount of labeled glucagon was 1.22 pmoles of cyclic 3',5'-AMP produced per 10 min; labeled material released from the membranes gave an activity of 1.32 ± 0.09 pmoles per 10 min. In two other experiments of this type, predicted and observed activities agreed within 10%. These findings indicate that glucagon forms a dissociable complex with its binding site and, when released by urea, retains its biological activity.

Effects of Temperature on Time Course of Uptake and Dissociation of Bound Glucagon—Labeled glucagon was taken up rapidly by the liver membranes, attaining a constant level within 10 min of incubation at either 0° or 30° (Fig. 11). Within the time of addition of membranes to the incubation medium and initiation of centrifugation (about 45 sec), about 30% of the total taken up at 10 min was bound to the membranes. It was assumed that separation of the membranes from the incubation medium was completed within seconds after centrifugation was begun. Nevertheless, it is apparent that the initial rate of binding was too rapid for measurement by the assay method used in this study. For this reason, it was not possible to evaluate the effects of temperature on the initial rate of uptake. However, it can be seen that the amount of glucagon bound in 10 min was reduced markedly when the membranes were incubated with labeled glucagon at 0°.

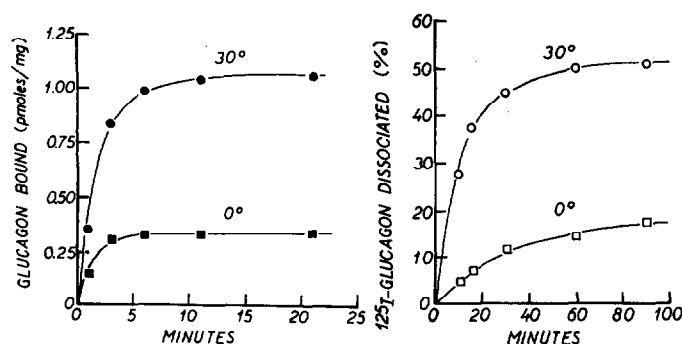The time course of dissociation of bound labeled glucagon was

FIG. 11 *(left)*. Effect of temperature on time course of binding of ^{125}I-glucagon to liver plasma membranes. Liver membranes (150 μg of protein) were incubated either at 0° or 30° in 0.750 ml of incubation medium containing 2.5% albumin, 4×10^{-9} M labeled glucagon (10^6 cpm per pmole), 1 mM EDTA, and 20 mM Tris-HCl, pH 7.6. Duplicate aliquots (50 μl) were withdrawn at indicated times and assayed for membrane-bound glucagon by Method A (see "Methods").

FIG. 12 *(right)*. Effect of temperature on time course of dissociation of bound ^{125}I-glucagon from plasma membranes. Incubations were carried out either at 0° or at 30° in two stages. In the first stage, plasma membranes (150 μg of protein) were incubated at 0.750 ml of incubation medium containing 2.5% albumin, 1 mM EDTA, 4×10^{-9} M labeled glucagon (10^6 cpm per pmole), and 20 mM Tris-HCl, pH 7.6. At 20 min, duplicate 50-μl aliquots were taken for measuring the amount of bound labeled hormone (Method A). The second stage of incubation was initiated by the addition of 5 μl of unlabeled glucagon to the remaining incubation mixture to give a final concentration of 5×10^{-6} M. At zero time (the moment of addition of unlabeled hormone) and at the times indicated, 50-μl samples were withdrawn and assayed for bound labeled glucagon by Method A (see "Methods"). Percentage of bound labeled glucagon dissociated was calculated from the difference in radioactivity bound in the two stages of incubation.

TABLE III

Effects of EDTA on steady state levels and dissociation of bound labeled glucagon

Liver membranes (25 μg of protein) were incubated in 0.125 ml of medium containing 2.5% albumin, 5×10^{-9} M ^{125}I-glucagon (10^6 cpm per pmole), and 20 mM Tris-HCl, pH 7.6. EDTA, when added, was 1 mM. Incubation time was 15 min, at 30°, in studies of association and dissociation. The latter was determined by the two-step procedure described in legend to Fig. 12. Results are the mean ± standard error of two experiments.

EDTA	Bound glucagon (steady state)	^{125}I-Glucagon dissociated
	pmoles/mg protein	*%*
−	1.3 ± 0.10	1 ± 1
+	1.3 ± 0.10	36 ± 3

investigated by first incubating the membranes with labeled glucagon at 0° or 30° for 15 min to ensure that a constant ("steady state")[4] level of bound glucagon had been attained. At this time, a 1000-fold excess of unlabeled glucagon was added to the incubation medium to prevent further uptake of labeled glucagon released from the membranes during further incubation at 0° or 30°. As illustrated in Fig. 12, labeled glucagon was released

[4] The term "steady state" is used in this study to define the time at which a constant amount of bound glucagon is attained. The usual kinetic definition of steady state does not apply since equilibrium between bound and free glucagon does not occur because of inactivation of glucagon during incubation.

rapidly either at 0° or 30° during the first 15 min of incubation, followed by a slower rate of dissociation. After 2 hours of incubation at 30°, a maximum of 50% of bound labeled glucagon was released to the incubation medium; less than 20% of the bound hormone was released during incubation at 0°. When plotted semilogarithmically, the time course of dissociation of bound glucagon at 30° did not demonstrate first order kinetics expected for simple dissociation of glucagon from its binding sites.

It was found subsequently that omission of EDTA from the incubation medium resulted in a marked decrease in the percentage of labeled glucagon dissociated from the membrane in the presence of unlabeled glucagon. An example of the effects of 1 mM EDTA on steady state levels of bound glucagon and the percentage of dissociation of bound labeled hormone after 15 min incubation with unlabeled hormone is shown in Table III. In these experiments, less than 2% of bound labeled glucagon dissociated within 15 min (in other experiments, not shown, less than 10% during 2 hours of incubation), in contrast to 35% dissociation in the presence of 1 mM EDTA. It will be noted that EDTA had no effect on the steady state level of bound glucagon. These findings indicated that the glucagon binding process is complex and are consistent with the previous results showing that glucagon forms a dissociable bond with its binding sites.

DISCUSSION

Labeled glucagon was used in this study to probe the nature of the initial reaction between glucagon and the adenyl cyclase system. It has not been established that iodinated glucagon used in the binding studies has the identical biological properties of glucagon or of mono-iodinated glucagon, both of which were shown to have equivalent effects on the liver membrane adenyl cyclase system. However, the following lines of evidence suggest that this material behaves as native glucagon. (a) Degradative products (not identified) of the iodination reaction did not bind to liver membranes or activate adenyl cyclase; (b) biological activity and binding of labeled glucagon were reduced in parallel and to the same extent by an inactivating process in the liver membrane preparation; and (c) binding of labeled glucagon was reduced specifically by native glucagon in proportion to the relative concentrations of labeled and unlabeled hormone in the incubation medium.

The finding that only glucagon, among the various peptide hormones or biologically inactive fragments of glucagon tested, reduced the binding of labeled glucagon indicates that the binding sites are specific for this hormone. The specific binding sites are finite in number (estimated 2.6 pmoles per mg of membrane protein) and have the same apparent affinity (4×10^{-9} M) for labeled glucagon as does the process by which adenyl cyclase is activated by glucagon. The affinity constant of the binding sites (or of the glucagon-activating process) could not be determined because of the rapid destruction or inactivation of the bulk of the glucagon during incubation with liver membranes. The specificity and other characteristics of the glucagon-inactivating process are under investigation.

The specificity for glucagon displayed by the binding sites and the finding that the range of concentrations over which the hormone binds and activates adenyl cyclase in these membranes are nearly identical suggest that the binding sites are components of the adenyl cyclase system. In this regard, fat cell ghosts, which contain an adenyl cyclase system that is activated by

glucagon over a range of concentration approximately 25-fold higher than the enzyme system in liver membranes, took up labeled glucagon to a lesser extent than the liver membranes. Studies of the characteristics of binding of glucagon to fat cell ghosts, currently in progress, should clarify whether the difference in binding is due solely to a decreased affinity of the binding sites for glucagon in these membranes.

It should be emphasized that the ultimate test for the function of the binding sites for glucagon will be derived from studies of the interaction between the purified components of the liver adenyl cyclase system. Specificity of binding, similarity in dose response and binding curves, and the inhibitory effects of agents that specifically inactivate the response of liver membrane adenyl cyclase to glucagon provide indirect, correlative-type evidence that the binding sites are related functionally as well as structurally to the glucagon-sensitive adenyl cyclase system.

It was of particular interest to find that phospholipase A and urea, agents that modify specifically the structure of lipids and proteins, decreased the binding of glucagon and activation of adenyl cyclase by the hormone. The inhibitory effects of phospholipase A suggest that the binding sites for glucagon may be lipoproteins, with lipids as essential structural components, and may be the basis for the inhibitory effects of phospholipase on the lipolytic response of isolated fat cells to several hormones that activate adenyl cyclase (24, 25). Consistent with this possibility are the previous findings (2) that digitonin inhibits the response of adenyl cyclase in fat cell ghosts to glucagon, secretin, ACTH, and epinephrine without altering the response of the enzyme system to fluoride ion. Digitonin-treatment of liver membranes also reduced the binding of glucagon by 70%; greater loss of the response of adenyl cyclase to glucagon raises the possibility that lipids may have functions in addition to the binding process. We have recently reported (26) that addition of specific phospholipids to digitonin-treated liver membranes results in partial restoration of the glucagon response of adenyl cyclase. Such studies may serve to distinguish between the lipoprotein character of the binding sites and a possible role of lipids in the activation process.

The inhibitory effects of 2 M urea on the binding of glucagon were reversed by simply diluting the concentration of the chaotropic agent. We have found recently that prior treatment of liver membranes with 0.5 M sodium perchlorate, a stronger denaturing agent than urea (27), results in irreversible loss of binding.[5] The observation that urea releases, quantitatively, bound glucagon in an active form indicates that glucagon, as has been reported (5) for the specific binding of ACTH to adrenal membranes, forms a noncovalent associative type bond with its binding sites in liver membranes. This is supported further by the finding that unlabeled glucagon exchanges with bound labeled glucagon. Whether binding is through hydrophobic interaction or through other forces remains unknown. Binding was dependent upon temperature, being reduced markedly at 0° relative to 30°. It is possible that temperature dependence of binding reflects alterations in the structure of the membranes as well as of the binding sites.

Uptake of glucagon by the liver membranes was very rapid, precluding measurements of the initial rate of association of the hormone with its binding sites. Since glucagon was also in-

activated to a considerable extent during the interval of time required to reach a constant or steady state level of bound hormone, it is likely that the observed time course of uptake of the hormone was influenced by the inactivating process. The time course of dissociation, which was measured under conditions in which a large concentration of glucagon (usually 5×10^{-6} M) was added, was probably not influenced by the inactivating process during the initial 5 or 10 min of incubation. During this time interval, EDTA stimulated the release of 35% of the bound labeled glucagon upon addition of unlabeled glucagon. This effect of EDTA suggests that other factors, in addition to the intrinsic binding forces between hormone and binding site, influence the binding reaction. EDTA was added to the incubation medium because of the previous observation (1) that it enhances the response of adenyl cyclase to glucagon in liver membranes. The relationship between the effects of EDTA on binding and hormonal response of adenyl cyclase remains unknown. In the following reports (7, 8), it will be shown that guanyl nucleotides also alter the binding of glucagon and the response of adenyl cyclase to glucagon.

In summary, glucagon binds to liver membranes at sites that are specific for glucagon, finite in number, and which display characteristics of a lipoprotein. Specificity of binding, similarity in range of concentrations over which the hormone binds and activates adenyl cyclase, and the correlations observed between loss of binding and loss of activation of adenyl cyclase by the hormone suggest that the binding sites are components of the glucagon-sensitive adenyl cyclase system in rat liver membranes. Subsequent studies (7, 8) deal further with the question of whether the binding sites for glucagon are functionally involved in the activation of adenyl cyclase.

REFERENCES

1. POHL, S. L., BIRNBAUMER, L., AND RODBELL, M., *J. Biol. Chem.*, **246**, 1849 (1971).
2. BIRNBAUMER, L., POHL, S. L., AND RODBELL, M., *J. Biol. Chem.*, **246**, 1857 (1971).
3. BIRNBAUMER, L., AND RODBELL, M., *J. Biol. Chem.*, **244**, 3477 (1969).
4. BÄR, H. P., AND HECHTER, O., *Proc. Nat. Acad. Sci. U. S. A.*, **63**, 350 (1969).
5. LEFKOWITZ, R. J., ROTH, J., PRICER, W., AND PASTAN, I., *Proc. Nat. Acad. Sci. U. S. A.*, **65**, 745 (1970).
6. TOMAZI, V., KORETZ, S., RAY, T. K., DUNNICK, J., AND MARINETTI, G. V., *Biochim. Biophys. Acta*, **211**, 31 (1970).
7. RODBELL, M., KRANS, H. M. J., POHL, S. L., AND BIRNBAUMER, L., *J. Biol. Chem.*, **246**, 1872 (1971).
8. RODBELL, M., BIRNBAUMER, L., POHL, S. L., AND KRANS, H. M. J., *J. Biol. Chem.*, **246**, 1877 (1971).
9. RODBELL, M., BIRNBAUMER, L., POHL, S. L., AND KRANS, H. M. J., *Acta Diabetol. Lat.*, **7** (Suppl. 1), 9 (1970).
10. NEVILLE, D. M., *Biochim. Biophys. Acta*, **154**, 540 (1968).
11. RODBELL, M., *J. Biol. Chem.*, **242**, 5744 (1967).
12. BIRNBAUMER, L., POHL, S. L., AND RODBELL, M., *J. Biol. Chem.*, **244**, 3468 (1969).
13. REISFELD, R. A., AND SMALL, P. A., JR., *Science*, **152**, 1253 (1966).
14. HUNTER, W. M., AND GREENWOOD, F. C., *Nature*, **194**, 495 (1962).
15. KAY, C. M., AND MARSH, M. M., *Biochim. Biophys. Acta*, **33**, 251 (1959).
16. YALOW, R. S., AND BERSON, S. A., in M. MARGOULIES (Edi-

[5] Unpublished observation.

tor), *Protein and polypeptide hormones*, Excerpta Medica Foundation, Amsterdam, 1969, p. 36.

17. BREWER, H. B., JR., KEUTMANN, II. T., POTTS, J. T., JR., REISFELD, R. A., SCHLUETER, R., AND MUNSON, P. L., *J. Biol. Chem.*, **243**, 5739 (1968).

18. LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., AND RANDALL, R. J., *J. Biol. Chem.*, **193**, 265 (1951).

19. UNGER, R. H., EISENTRAUT, A. M., McCALL, M. S., AND TIMM, D. L., *J. Clin. Invest.*, **40**, 1280 (1961).

20. JUNEK, H., KIRK, K. L., AND COHEN, L. A., *Biochemistry*, **8**, 1844 (1969).

21. STAUB, A., SINN, L., AND BEHRENS, O. K., *J. Biol. Chem.*, **214**, 619 (1955).

22. SWANN, J. C., AND HAMMES, G. G., *Biochemistry*, **8**, 1 (1969).

23. FOLK, J. E., AND COLE, P. W., *J. Biol. Chem.*, **240**, 193 (1965).

24. RODBELL, M., AND JONES, A. B., *J. Biol. Chem.*, **241**, 140 (1966).

25. BLECHER, M., *Biochim. Biophys. Acta*, **187**, 380 (1969).

26. BIRNBAUMER, L., POHL, S. L., KRANS, H. M. J., AND RODBELL, M., *Advan. Biochem. Psychopharmacol.*, **3**, 185 (1970).

27. HATEFI, Y., AND HANSTEIN, W. G., *Proc. Nat. Acad. Sci. U. S. A.*, **62**, 1129 (1969).